IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: HUMAN LYMPHOCYTE VACCINE ADJUVANT
Kenichiro Hasumi et al.	:
Application Serial Number 10/783,259	: Examiner Louise Wang Zhiying Humphrey
Filing Date: February 20, 2004	: Attorney Docket Number 358690-00005-1
	: Art Unit 1648

DECLARATION UNDER 37 C.F.R. § 1.132

Eckert Seamans Cherin & Mellott, LLC
U.S. Steel Tower
600 Grant Street, 44th Floor
Pittsburgh, Pennsylvania 15219

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Sir:

I, Dean L. Mann, declare as follows:

1. I am one of the named inventors of the invention described and claimed in the above-identified patent application.

2. I am a citizen of the United States and reside at 22 S. Greene Street, Baltimore, MD 21201. I attended Goshen College, Goshen, Indiana and graduated in 1956 with a B.S. in science. I attended St. Louis University School of Medicine, St. Louis MO and graduated in 1963 with an M.D. From 1963 to 1964, I was a medical intern at St. Louis University School of Medicine. From 1964-1966, I was an Assistant Resident at St. Louis University School of Medicine. From 1966-1968 I held an NIH Postdoctoral Fellowship in Immunology. From 1968 to 1983, I was a Medical Officer at the NIH, NCI, Division of Cancer Biology and Diagnosis in Bethesda, MD. From 1983-1987, I was a Medical Officer at the NIH, NCI, Division of Cancer Etiology, Laboratory of Human Carcinogenesis in Bethesda, MD. From 1984-1987, I was head of the Biochemical Epidemiology Section, Laboratory of Human Carcinogenesis in Bethesda, MD. From 1987 to 1996, I was Chief of Immunogenetics Section, Laboratory of Viral Carcinogenesis, DBS, NCI-FCRDC in Frederick, MD. From 1996 through the present, I have been Professor in the Department of

Pathology at the University of Maryland School of Medicine. In addition, from 1996-2005, I was Associate Director of the American Red Cross National Histocompatibility Testing Laboratory of the University of Maryland; and from 1996 through the present I have served as head of the Division of Immunogenetics, Department of Pathology, University of Maryland, Baltimore School of Medicine. A copy of my curriculum vitae is attached hereto as Exhibit A.

3. I have read and am thoroughly familiar with the contents of the above-captioned patent application as well as the contents of the Office Action dated August 8, 2006 and the Amendment dated January 8, 2007, filed concurrently herewith. As a specialist in the field of immunology and immunogenetics, and on the basis of this review, it is my well-considered opinion that the invention is more than adequately enabled for the entire scope of the claimed invention and that one skilled in the art would be able to practice the claimed invention in mammals *in* vivo as well as *in vitro* without undue experimentation. Evidence for this attestation is provided below.

4. As fully described in the accompanying paper (attached hereto as Exhibit B), an investigation was undertaken by the inventors of the instant application to determine whether the co-administration of vaccines with the activated lymphocyte conditioned media (LCM) of the present invention enhances T cell and antibody immune responses in vivo in non-human primates.

5. The methods of the study included, in brief, taking cryopreserved peripheral blood mononuclear cells (PBMCs) from immunized macaques and washing them in RPMI-1640 containing 20% bovine AB serum (bAB). The PBMCs were resuspended in cRPM. 10^5 cells/well were cultured with 10 and 20μg/ml Hepatitis A Vaccine, Rabies Vaccine, Tetanus and Diphtheria Toxoids and prostate specific antigen (PSA) highly pure antigen in coated Elispot plates. As a negative control, cells were cultured in cRPMI alone. Cells were stimulated with 10μg/ml Con A as a positive control. All conditions were plated in triplicate in a volume of 100μl/well and incubated for 72 hours at 37°C in 5% CO_2. Elispot plates were prepared by standard method as follows. 96-well nitrocellulose-bottom plates were coated with 100μl/well of anti-human/monkey IFNγ at a concentration of 15μg/ml and incubated at 4°C overnight. The following day, the plates were washed six times with 1X PBS and blocked with 100μl/well of cRPMI. PBMC were plated in triplicate in a volume of 100μl/well and incubated for 72 hours at 37°C in 5% CO_2. ELISPOT plates were washed six times with PBS and incubated for 3 hours at 25°C with 100μl/well of biotinylated anti-human/monkey IFNγ at a concentration of 1μg/ml. Plates were washed six times in PBS and

incubated for 1 hour with 100µl/well of Streptavidin-HRP at 25°C. ELISPOT plates were washed a final time in 1X PBS and developed for 30 minutes with 100µl/well of peroxidase substrate AEC kit, followed by rinsing in tap water. Plates were stored over night in the dark at room temperature, and spots were counted using a VersaScan microplate reader. The mean totals of IFNγ spot-forming cells (SFC) in triplicate wells were determined and expressed as numbers of SFC per 1 x 10^5 PBMC. Sera from immunized macaques were screened for antibodies by ELISA. Antibody titers to hepatitis A were determined by HEPAVASE A-96. Antibody titers to diphtheria and tetanus toxoids were quantified by Diphtheria IgG ELISA and Tetanus IgG ELISA.

6. The results are shown in Exhibit B in Table 1, Figure 1 and Figure 3. Table 1 shows the concentration of pro-inflamatory cytokines and chemokines in the activated LCM that was co-injected with the vaccines to determine if the LCM enhanced T cell and antibody responses in non-human primates to these antigens. Figure 1 shows the time-lines of administration of the vaccines alone and the vaccine co-injected with LCM. This figure also records the days of procurement of cells for examination of T cell response and serum for antibody titer.

7. The recall response to the vaccines in PBMCs from monkeys infected with the vaccine alone or combined with LCM are shown in Fig.2 of Exhibit B hereto. (Due to a freezer accident. the cells and serum obtained from the first 5 blood draws from the control monkeys were lost). Nonetheless, comparison of responses in cells obtained at day 35 in both groups demonstrated residual T cell memory only in some of the animals receiving the vaccines combined with activated LCM. Antibody titers also were greater at this time point and appeared to be sustained at higher levels in the monkeys receiving the vaccines plus activated LCM. Indeed, one of the most convincing pieces of data that LCM acts to enhance immunity to a vaccine is the T cell responses observed to PSA in male monkeys. This is because PSA in non-human primates is closely related in its genetic sequence to the human counterpart that was used as an immunogen in this study.

8. In conclusion, I declare that the results from the above-described investigation clearly show that the activated LCM of the present invention can be used *in vivo* as an adjuvant in vaccines where an enhanced immune response is desired.

9. I declare further that all statements made herein of my own knowledge are true and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of

Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application and any patent issuing thereon.

Dean L. Mann, M.D.

01/05/07
(DATE)

CURRICULUM VITAE

Dean L. Mann, M.D.
Professor and Head, Division of Immunogenetics
University of Maryland, School of Medicine

Date January 26, 2006

Personal Information

Business Address: Division of Immunogenetics
Department of Pathology
University of Maryland, School of Medicine, and

Program in Oncology
The University of Maryland
Marlene and Stewart Greenebaum Cancer Center

Office Address: University of Maryland Medical System
Department of Pathology, Division of Immunogenetics
22 S. Greene Street
Baltimore, MD 21201

Phone Number: (410) 328-5512

Education

1956	B.A.	Science	Goshen College, Goshen, Indiana
1963	M.D.		St. Louis University School of Medicine St. Louis, MO

Internship (medical)

1963-1964 St. Louis University Hospital
St. Louis, MO

Residency

1964-1966 Assistant Resident, St. Louis University Hospital
St. Louis, MO

Fellowships

1966-1968 NIH Postdoctoral Fellowship in Immunology

Medical Licensures

Virginia
Maryland (Active): #D0050237

Major Research Interests: Immunogenetics; cell surface antigens; structure/function of major histocompatibility gene products. Immunobiology of AIDS and cancer.

Military Service: None

Employment History

1968-1983	Medical Officer, NIH, NCI, Division of Cancer Biology and Diagnosis, Bethesda, MD
1983-1987	Medical Officer, NIH, NCI, Division of Cancer Etiology, Laboratory of Human Carcinogenesis, Bethesda, MD
1984-1987	Head, Biochemical Epidemiology Section, Laboratory of Human Carcinogenesis, Bethesda, MD
1987-1996	Chief, Immunogenetics Section, Laboratory of Viral Carcinogenesis, DBS, NCI-FCRDC, Frederick, MD
1996-present	Professor, Department of Pathology, University of Maryland,Baltimore, School of Medicine

Major Academic Tasks

1996-2005	Associate Director, American Red Cross National Histocompatibility Testing Laboratory of the University of Maryland
1996-present	Head, Division of Immunogenetics, Department of Pathology, University of Maryland, Baltimore, School of Medicine. The Division has a clinical laboratory that provides histocompatibility testing for transplant services at the University Hospital.

Professional Memberships

American Association for Histocompatibility and Immunogenetics
American Federation for Clinical Research
American Association of Immunologists
Transplantation Society
American Society for Clinical Investigation

Honors, Awards

Alpha Omega Alpha
Distinguished Visiting Professor, University of Vienna,
School of Medicine, Vienna, Austria

Institutional Service
Member. External Advisory Board, Myeloma Institute for Research and Therapy, University of Arkansas, School of Medicine

Administrative Service

National Service
Member. NIAID Vaccine Study Section

Editorial Boards

Reviewer:

1985-present	AIDS
"	Blood
"	Clinical Immunology and Immunopathology
"	Journal of AIDS
"	Journal of AIDS and Human Retrovirology
"	Journal of Immunology
"	Nature Medicine
"	Science
"	Journal of Infectious Disease
"	Immunology and Immunopathology
"	Carcinogenesis
2001-present	Immuno-genetics
"	Human Immunology
2002-present	PNAS

Teaching Responsibilities

2005	Currently mentoring 3 graduate students and 1 post-doctoral fellow in my research laboratory and serving on thesis committees of 3 additional graduate students. One 1st year resident rotated through the lab for several months during 2004.

Grant Support

Active

Project Number/PI:	1R01HL69057-01A1/Jeffery Hasday
Dates of Approved Project:	3/1/03-2/28/07
Total Direct Costs:	$1,250,000
Total Indirect Costs:	$291,250
Effort:	10%
Role:	Co-investigator
Source:	NHLBI/NIH
Title of Project:	"Mechanisms of Fever-Enhanced Hyperoxic Lung Injury"

The major goal of this project is to perform a structure function analysis of the heat-shock-activated transcription factor HSF1 relevant to its TNF repressing activity, to define the molecular basis of its interaction with the TNF promotor. and to determine how it is modified by protein kinases that are activated by bacterial endotoxin.

Project Number/PI:	Mann, D.L., M.D.
Dates of Approved Project:	10/2/00-present
Total Direct Costs:	$9.550.00
Total Indirect Costs:	$0
Source:	AP Intramural
Title of Project:	"Determination of the Role of MICA. MICB in Allograft Rejection"

The objective of this project is to determine the expression of the non-classical HLA Class I-like molecules in allografted tissue and their relationship to rejection.

Project Number/PI:	5U01-AI054641-02/Mann, D.L. M.D.
Dates of Approved Project:	5/15/03-4/30/07
Total Direct Costs:	$1,498,371
Total Indirect Costs:	$726,709
Effort:	20%
Source:	The Dow Chemical Co./ NIAID
Title of Project:	"Non-invasive Plant Virus-particle Based Anthrax Vaccines"

To assess the *in vitro* and *in vivo* immunogenicity of the plant viral construct expressing anthrax P protein peptides. These studies will confirm the efficacy of plant viral particle based anthrax vaccine.

Project Number/PI:	HASUMI/Mann, D.L., M.D.
Dates of Approved Project:	11/17/03-6/30/06
Total Direct Costs:	$397,261
Total Indirect Costs:	$79,452
Effort:	10%
Source:	Hasumi International Research Foundation
Title of Project:	"Development of Dendritic Cell Based Vaccines for Cancer Immunotherapy"

The overall objective of this proposal is to establish a preclinical model that will address: substances that are produced by the tumors that can be recognized by the host immune system need to be identified and an effective means of delivery of the immunogen.

Project Number/PI:	1R21CA101356-01A2/Dr. Aaron Rapoport (Dr. Mann: Co-P.I.)
Dates of Approved Project:	8/5/04-7/31/06
Total Direct Costs:	$225,000
Total Indirect Costs:	$0
Effort:	15%
Source:	NCI/NIH
Title of Project:	"Immune Responses to T cell Expansion + PCV Immunization"

This is a quick trial for novel cancer therapies to analyze the matter of immune reconstitution. measure T-cell response and assay for the development of T-cell populations.

Project Number/PI:	Mann, D.L., M.D.
Dates of Approved Project:	9/1/04-8/31/06
Total Direct Costs:	$269,360
Total Indirect Costs:	$130,640
Effort:	3%
Source:	Fraunhofer USA/ DOD
Title of Project:	"Targets of the Immune Response Vaccines to Combat Infectious Pathogens"

To determine the capacity of candidate vaccines to generate immune responses in human lymphoid cells in vitro. these studies are designed to determine the extent to which these component vaccines will be recognized by the majority of the individuals in the human population.

Project Number/PI:	1U24CA11509-01/S.A. Stass, M.D., P.I. (Dr. Mann, Co-investigator)
Dates of Approved Project:	3/21/05-2/28/10
Total Direct Costs:	$965,828
Total Indirect Costs:	$468,427
Effort:	Yr 2 Effort Pending
Source:	NIH
Title of Project:	"University of Maryland Biomarker Reference Laboratory"

Continue the national network that has the responsibility for the development, evaluation. and validation of biomarkers for earlier cancer detection and risk assessment.

Project Number/PI:	1R01AI067503-01
Dates of Approved Project:	8/15/05-1/31/07
Annual Direct Costs:	$175,090
Annual Indirect Costs:	$84,918
Effort:	3%
Source:	NIH/NIAID
Title of Project:	"Immune Reconstitution in Nonhuman Primates"

We will test the central hypothesis that uropathogenic E. coli and P. mirabilis regulate the balance between motility and adherence.

Pending

Project Number/PI:	5U01-AI054641-02/Mann, D.L. M.D.
Dates of Approved Project:	6/1/05-4/30/06
Total Direct Costs:	$93.808 (Supplement)
Effort:	%

Source:	The Dow Chemical Co./ NIAID
Title of Project:	"Non-invasive Plant Virus-particle Based Anthrax Vaccines"

To assess the *in vitro* and *in vivo* immunogenicity of the plant viral construct expressing anthrax P protein peptides. These studies will confirm the efficacy of plant viral particle based anthrax vaccine.

Project Number/PI:	PAR-05-152/Shiraz I. Mishra, MBBS. Ph.D. (Dr. Mann, Co-investigator)
Dates of Approved Project:	7/1/06-6/30/09
Total Direct Costs:	$400,000
Effort:	1%-Yrs 1 & 2: 2%-Yr 3
Source:	NIH/NCCAM
Title of Project:	Mindfulness Based Stress Reduction Program for Breast Cancer Survivors

The pilot poject explores the feasibility of implementing a mind-body medicine therapy, the Mindfulness Based Stress Reduction Program, among African American and Caucasian post-treatment, early stage breast cancer survivors. In addition, the pilot project assesses whether the program equally impact African American and Caucasian women on outcomes such as quality of life, psychological distress, locus of control, symptoms of stress, and immune function.

Project Number/PI:	Mann, D.L. M.D.
Dates of Approved Project:	3/31/06-3/30/08
Total Direct Costs:	$281,143
Effort:	5%
Source:	Booz Allen Hamilton, Inc.
Title of Project:	Professional Support Services–NIAID Flow Cytometry Personnel

Completed Research Support

Project Number/PI:	#1RO1A141951-01/Kaslow
Dates of Approved Project:	09/01/97-08/31/00
Annual Direct Costs:	$42,089
Effort:	5%
Source:	NIAID/NIH (Univ Alabama at Birmingham)
(Subcontract)	University of Maryland, Dr. Mann Co-investigator
Title of Project:	"Genetic Factors in the Epidemiology of HIV-1 Infection"

This project investigated the role of alleles of the major histocompatibility complex Class I and Class II genes on disease progression in an HIV-1 infected cohort.

Project Number/PI:	DAMD17-98-1-8466/Mann, D.L., M.D.
Dates of Approved Project:	07/01/98-12/31/01
Annual Direct Costs:	$125,000
Effort:	15%
Source:	USMRMC
Title of Project:	"Prostate Tumor Antigen Discovery: Development of a Novel Genetic Approach"

To investigate parameters of immunologic response to prostate cancer cells in order to establish a rational immunotherapeutic treatment modality using potent antigen presenting dendritic cells.

Project Number/PI:	5-R24-CA82888-03/Mann, D.L., M.D.
Dates of Approved Project:	8/1/99-7/31/03
Annual Direct Costs:	$150.000
Effort:	15%
Source:	NCI, NIH
Title of Project:	"Flow Cytometry Applications in Cancer Biology"

The goal of this project is to provide state-of-the-art flow cytometry equipment, trained personnel, and advice and direction for the use of this equipment in studies of cancer biology.

Project Number/PI:	1-R01-GM58721-01A2/DeClaris, N., Ph.D. (Mann, D.L., M.D., Co-PI)
Dates of Approved Project:	9/1/00-8/31/03
Annual Direct Costs:	$102,038
Effort:	10%
Source:	NIH (U of Texas, M.D. Anderson Cancer Center (Subcontract))
Title of Project:	"Robust Generalization in MHC Peptide Binding Models"

The goal of this project was to explore the potential of an innovative feature space geometric approach for the design and implementation of MHC peptide binding models. Dr. Mann designs experiments to test the predicted peptides for function.

Project Number/PI:	5U01-CA-069854-08/Karp (Mann, D.L., MD. Investigator)
Dates of Approved Project:	3/1/02-2/28/03
Annual Direct Costs:	$399,076
Effort:	5%
Source:	NCI, NIH
Title of Project:	"Phase I Trials of Anti-cancer Agents"

Dr. Mann supervises flow cytometry studies that are used to monitor patient responses to chemotherapeutic agents in clinical Phase I trials.

Project Number/PI:	Mann, D.L., M.D.
Dates of Approved Project:	7/1/02-6/30/04
Annual Direct Costs:	$183,128
Effort:	15%
Source:	Hasumi International Research Foundation
Title of Project:	"Development of Dendritic Cell-Based Vaccines for Cancer Immunotherapy"

The overall objective of this proposal is to establish a preclinical model that will address: substances that are produced by the tumors that can be recognized by the host, immune system needs to be identified, and an effective means of delivery of the immunogens must be developed.

Publications

Journal Articles (Peer Reviewed)

1. Mann, D.L., Sites, M.L., Donati, R.M., & Gallagher, N.I. Erythropoietic stimulating activity during the first ninety days of life. Proceedings of the Society for Experimental Biology and Medicine, 118: 212-217, 1965.

2. Mann, D.L., Donati, R.M., & Gallagher, N.I. Erythropoietin assay and ferrokinetic measurements in anemic uremic patients. JAMA, 194: 1321-1324, 1965.

3. Mann, D.L., Donati, R.M., & Gallagher, N.I. Effect of renin, angiotensin II and aldosterone on erythropoiesis. Proceedings of the Society for Experimental Biology and Medicine, 121: 1152-1157, 1966.

4. Mann, D.L., Gallagher, N.I., & Donati, R.M. Erythrocytosis and primary aldosteronism. Annals of Internal Medicine, 66: 335-339, 1967.

5. Mann, D.L., Donati, R.M., & Gallagher, N.I. Relationship of renal mass to erythropoietin production. Laboratory Investigation, 19: 406-411, 1968.

6. Mann, D.L., Rogentine, G.N. Jr., Fahey, J.L., & Nathenson, S.G. Solubilization of human leukocyte membrane isoantigens. Nature, 217: 1180-1181, 1968.

7. Mann, D.L., Granger, H., & Fahey, J.L. Use of insoluble antibody for the determination of small amounts of immunoglobulins. Journal of Immunology, 102: 618-624, 1969.

8. Mann, D.L., Rogentine, G.N. Jr., Fahey, J.L., & Nathenson, S.G., Solubilization, properties and molecular separation of HL-A alloantigens. Transplantation Proceedings, 1: 494-497, 1969.

9. Mann, D.L., Rogentine, G.N. Jr., Fahey, J.L., & Nathenson, S.G. Molecular heterogeneity of human lymphoid (HL-A) alloantigens. Science, 163: 1460-1462, 1969.

10. Mann, D.L., Rogentine, G.N. Jr., Fahey, J.L., & Nathenson, S.G. Human lymphocyte membrane (HL-A) alloantigens: Isolation, purification and properties. Journal of Immunology, 103: 282-292, 1969.

11. Fahey, J.L., Mann, D.L., Asofsky, R., & Rogentine, G.N. Jr. Recent progress in human transplantation immunology. Annals of Internal Medicine, 71: 1177-1195, 1969.

12. Mann, D.L. & Nathenson, S.G. Comparisons of soluble human and mouse transplantation antigens. Proceedings of the National Academy of Sciences USA. 64: 1380-1387, 1969.

13. Mann, D.L., Fahey, J.L., & Nathenson, S.G. Molecular comparisons of papain solubilized H-2 and HL-A alloantigens. Histocompatibility Testing. Copenhagen. Munksgaard. pp. 461-467. 1970.

14. Graff, R.G., Mann, D.L., & Nathenson, S.G. Immunogenic properties of papain-solubilized H-2 alloantigens. Transplantation. 10: 59-64. 1970.

15. Nathenson, S.G., Shimada, A., Yamane, K., Maramatsu, T., Cullen, S., Mann, D.L., Fahey, J.L., &

Graff, R.G. Biochemical properties of papain solubilized murine and human histocompatibility alloantigens. Federation Proceedings, 29: 2026-2033, 1970.

16. Leventhal, B.G., Rogentine, G.N. Jr., & Mann, D.L. Sensitization to water soluble transplantation antigens. Transplantation. 3: 243-245, 1971.

17. Mann, D.L. & Fahey, J.L. Properties of HL-A alloantigens solubilized by chemical techniques. Transplantation Proceedings, 3: 234-237, 1971.

18. Rosenberg, E.B., Mann, D.L., Hill, J.J., & Fahey, J.L. Prolonged allograft survival in mice pretreated with soluble transplantation antigens. Transplantation, 12: 402-405, 1971.

19. Merritt, C.B., Mann, D.L., & Rogentine, G.N. Jr. Cytotoxic antibody in human graft versus host disease. Nature, 232: 638-641, 1971.

20. Mann, D.L. & Fahey, J.L. Transplantation antigens. Annual Review of Microbiology, 25: 679-710, 1971.

21. Einstein, A.B., Mann, D.L., Gordon, H.G., Trapani, R.J., & Fahey, J. Heterologous antisera against specific HL-A alloantigens. Transplantation, 12: 299-304, 1971.

22. Einstein, A.B., Mann, D.L., Gordon, H.G., & Fahey, J.L. The immune reactivity of heterologous antisera against solubilized lymphoid cell membrane component. Tissue Antigens, 1: 209-218, 1971.

23. Einstein, A.B., Mann, D.L., Ficker, S., & Terry, W.D. Antiserum to soluble lymphoid membrane preparation stimulates human leukocyte DNA synthesis. Journal of Immunology, 107: 1205-1208. 1971.

24. Whisnant, J., Mann, D.L., Rogentine, G.N. Jr., & Robbins, J. Human cell surface structures related to haemophilus influenze type B disease. Lancet, 2: 895-898, 1971.

25. Mann, D.L., Halterman, R., Rogentine, G.N. Jr., & Leventhal, B. Detection of an acute leukemia associated antigen. Science, 174: 1136-1137, 1971.

26. Mann, D.L. Effect of enzyme inhibition on the solubilization of HL-A antigen with 3M KCl. Transplantation, 14: 398-401, 1972.

27. Amos, B.D., Bodmer, W.F., Ceppellini, R., Condliffe, P.G., Dausett, J., Fahey, J.L., Goodman, H.C., Klein, G., Klein, J., Lilly, F., Mann, D.L., McDevitt, H., Nathanson, S., Palm, J., Reisfeld, R.A., Rogentine, G.N., Sanderson, A.R., Shreffler, D.C., Simonsen, M., & van Rood, J.J. Biologic significance of histocompatibility antigens. Federation Proceedings. 31: 1087-1104. 1972.

28. Halterman, R., Leventhal, B.G., & Mann, D.L. An acute leukemia associated antigen: Clinical correlation. New England Journal of Medicine, 287: 1272-1274. 1972.

29. Gavin, J.R., Mann, D.L., Buell, D.N., & Roth, J. Preparation of solubilized insulin receptors from human lymphocytes. Biochemical and Biophysical Research Communication. 49: 870-876, 1972.

30. Mann, D.L., Halterman, R., & Leventhal, B.G. Cross reactive antigens on human cells infected

with Rauscher leukemia virus and on human acute leukemia cells. Proceedings of the National Academy of Sciences, 70: 495-497, 1973.

31. Yust, I., Wunderlich, J.R., Mann, D.L., & Buell, D.N. Cytotoxicity mediated by human lymphocyte dependent antibody in a rapid assay with adherent target cells. Journal of Immunology, 110: 1672-1681, 1973.

32. Mann, D.L., Halterman, R., & Leventhal, B. Acute leukemia associated antigens. Cancer, 34: 1446-1451, 1974.

33. Springer, T.A., Strominger, J.L., & Mann, D.L. Partial purification of detergent soluble HL-A antigen and its cleavage by papain. Proceedings of the National Academy of Sciences USA, 71: 1539-1543, 1974.

34. Yust, I., Wunderlich, J.R., Mann, D.L., & Terry, W.D. Identification of lymphocyte dependent antibody in sera from multiply transfused patients. Transplantation, 18: 99-107, 1974.

35. Yust, I., Wunderlich, J., Mann, D.L., Leventhal, B., Yankee, R., & Graw, R. Human lymphocyte dependent antibody mediated cytotoxicity and direct lymphocyte cytotoxicity against non-HL-A antigens. Nature, 249: 263-265, 1974.

36. Strominger, J.L., Cresswell, P., Grey, H., Humphreys, R.H., Mann, D.L., McCune, J., Parham, P., Robb, R., Sanderson, A.R., Springer, T.A., Terhorst, C., & Turner, M.J. The immunoglobulin-like structure of human histocompatibility antigens. Transplantation Reviews, 21: 126-143. 1974.

37. Robb, R.J., Humphreys, R.E., Ruller, T.C., Mann, D.L., & Strominger, J.L. Rabbit antisera to papain-solubilized HL-A antigens. Transplantation, 19: 445-447, 1975.

38. Yust, I., Smith, R.W., Dickler, H.B., Wunderlich, J., & Mann, D.L. Human lymphocyte dependent antibody mediated cytotoxicity: Adherence of lymphocytes to antibody treated target cells. Cellular Immunology, 18: 176-186, 1975.

39. Mann, D.L. An approach to the development of antisera to tumor associated antigens: Experience with acute leukemia and melanoma. Monograph: Symposium on Immunologic Reactions to Melanoma Antigens. Behring Institute Mitteilungen, 56: 103-106, 1975.

40. Mann, D.L., Leventhal, B., & Halterman, R. Human antiserums detecting leukemia associated antigens on autochthonous tumor cells. Journal of the National Cancer Institute, 54: 345-347, 1975.

41. MacDonald, J.S., Wunderlich, J.R., Yust, I., Mann, D.L., & Yankee, R.A. Complement-dependent and cell-dependent antiplatelet humoral antibody in sera from multiply transfused patients. Clinical and Experimental Immunology, 21: 259-266. 1975.

42. Miller, J.L., Mann, D.L., & Yust, I. Separation of complement dependent and lymphocyte dependent activity in human sera. European Journal of Immunology, 5: 546-548. 1975.

43. Mann, D.L., Abelson, L., Harris, S., & Amos, D.B. Detection of antigens specific for "B" lymphoid cultured cell lines with human alloantisera. Journal of Experimental Medicine, 142: 84-89. 1975.

44. Sacks, K.L., Olweny, C., Mann, D.L., Simon, R., Johnson, G.E., Poplack, D.G., & Leventhal, B.D. A clinical trial of chemotherapy and RAJI immunotherapy in advanced acute lymphatic leukemia (ALL): Clinical and laboratory observations. Cancer Research, 35: 3715-3720, 1975.

45. Mann, D.L., Abelson, L., Henkart, P., Harris, S.D., & Amos, D.B. Specific human B lymphocyte alloantigens (L-B) linked to HL-A. Proceedings of the National Academy of Sciences, 72: 5103-5106, 1975.

46. Pendergrass, T.W., Stoller, R.G., Mann, D.L., Halterman, R.H., & Fraumeni, J.F. Acute myelocytic leukemia and leukemia associated antigens in sisters. Lancet, ii: 429-431, 1975.

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Methods

Antigen Stimulation of Immunized Macaque PBL

Cryopreserved PBMC from immunized macaques were washed in RPMI-1640 containing 20% bovine AB serum (bAB), and resuspended in cRPMI[RPMI-1640 supplemented with 10% bAB, 2mM L-glutamine, 1% Penicillin-Streptomycin Solution, and 20mM Hepes Buffer (Invitrogen)]. 10^5 cells/well were cultured with 10 and 20μg/ml Hepatitis A Vaccine (SmithKline Beecham Pharmaceuticals, Philadelphia, PA), Rabies Vaccine, Tetanus and Diphtheria Toxoids (Aventis Pasteur Inc, Swiftwater, PA) and PSA highly pure antigen (Fitzgerald Industries International Inc, Concord, MA) in coated Elispot plates. As a negative control, cells were cultured in cRPMI alone. Cells were stimulated with 10μg/ml Con A (Sigma) as a positive control. All conditions were plated in triplicate in a volume of 100μl/well and incubated for 72 hours at 37°C in 5% CO_2. Elispot plates were prepared by standard method as follows.

ELISPOT Assay

96-well nitrocellulose-bottom plates (Multiscreen-HA, Millipore, Molsheim, France) were coated with 100μl/well of anti-human/monkey IFNγ at a concentration of 15μg/ml (GZ-4, Mabtech, Nacka, Sweden) and incubated at 4°C overnight. The following day, the plates were washed six times with 1X PBS (Invitrogen) and blocked with 100μl/well of cRPMI. PBMC were plated in triplicate in a volume of 100μl/well and incubated for 72 hours at 37°C in 5% CO_2.

ELISPOT plates were washed six times with 1X PBS and incubated for 3 hours at 25°C with 100μl/well of biotinylated anti-human/monkey IFNγ (7-B6-1, Mabtech) at a concentration of 1μg/ml. Plates were washed six times in 1X PBS and incubated for 1

hour with 100µl/well of Streptavidin-HRP (Mabtech) at 25°C. ELISPOT plates were washed a final time in 1X PBS and developed for 30 minutes with 100µl/well of peroxidase substrate AEC kit (Vector Laboratories, Burlingame, CA), followed by rinsing in tap water. Plates were stored over night in the dark at room temperature, and spots were counted using a VersaScan microplate reader (Velocity 11, Palo Alto, CA). The mean totals of IFNγ spot-forming cells (SFC) in triplicate wells were determined and expressed as numbers of SFC per 1 x 10^5 PBMC.

Determination of Antibody Titers by ELISA

Sera from immunized macaques were screened for antibodies by ELISA. Antibody titers to hepatitis A were determined by HEPAVASE A-96 (Labexim International, Lengau, Austria). Antibody titers to diphtheria and tetanus toxoids were quantified by Diphtheria IgG ELISA and Tetanus IgG ELISA (IBL, Hamburg, Germany) respectively. Methods followed manufacturer's instruction.

Results

Table 1 shows the concentration of pro-inflamatory cytokines and chemokines in the LCM (activated lymphocyte media) that was co-injected with the vaccines to determine if the LCM enhanced T cell and antibody responses in non-human primates to these antigens. Figure 1 shows the time-lines of administration of the vaccines alone and the vaccine co-injected with LCM. This figure also records the days of procurement of cells for examination of T cell response and serum for antibody titer.

The results of the recall response to the vaccines in peripheral blood mononuclear cells from monkeys infect with the vaccine alone or combined with LCM are shown in Fig.2 Due to a freezer accident the cells and serum obtained from the first 5 blood draws from the control monkeys were lost. Even so comparison of responses in cells obtained at day 35 in both groups demonstrates residual T cell memory only in some of the animals receiving the vaccines combined with LCM. Antibody titers were also greater at this time

point and appeared to be sustained at higher levels in the monkeys receiving the vaccines plus LCM. One of the most convincing pieces of data that LCM acts to enhance immunity to a vaccine is the T cell responses observed to PSA (prostate specific antigen) given that these were male monkeys and that PSA in non-human primates is closely related in sequence to the human counterpart that was used as an immunogen in this study. The results suggests that LCM might be useful as an adjuvant in Cancer vaccines where the immune response desired is directed at self antigens.

Figure 1: Control Macaques Timeline

	Injection									
HepA	+	+	+							
TDT	+	+	+							
Rabies	+	+	+							
PSA	+	+	+							
	0	7	14	21	28	35	42	49	56	**Day**
Cells	+					+	+	+	+	
Serum	+					+	+	+	+	
	Samples Collected									

LCM Treated Macaques Timeline

	Injection									
HepA	+	+	+							
TDT	+	+	+							
Rabies	+	+	+							
PSA	+		+							
	0	7	14	21	28	35	42	49	56	**Day**
Cells	+	+	+	+	+	+	+	+	+	
Serum	+	+	+	+	+	+	+	+	+	
	Samples Collected									

Table 1: Concentration of LCM used in Macaque study = 325ng/ml

Cytokine/Chemokine Concentrations of Pooled LCM Injected into Macaques			
	ng/ml	*ng/injection site*	*total ng/injection*
GM-CSF	310	93	372
IL-4	2.5	0.75	3
IL-5	1.5	0.45	1.8
IL-8	4.3	1.29	5.2
IL-10	3.2	0.96	3.8
MCP-1	3.7	1.11	4.4
IL-1a	0.228	0.07	0.274
IL-1b	0.364	0.11	0.437
IL-12p40	0.313	0.09	0.376

0.3ml LCM (97.5ng) was mixed with individual vaccines prior to injection. The vaccine/LCM mixture was then injected IM at four separate sites (right and left arm and thigh).

Figure 3: Antibody Response